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Anna Elisa Laria, Sebastiano Messineo, Biagio Arcidiacono, Mariaconcetta Varano, Eusebio Chiefari, Robert K. Semple, Nuno Rocha, Diego Russo, Giovanni Cuda, Marco Gaspari, Antonio Brunetti,* and Daniela P. Foti**

In the obese state, as adipose tissue expands, adipocytes become hypoxic and dysfunctional, leading to changes in the pattern of adipocyte-secreted proteins. To better understand the role of hypoxia in the mechanisms linked to obesity, we comparatively analyzed the secretome of murine differentiated 3T3-L1 adipocytes exposed to normoxia or hypoxia for 24 h. Proteins secreted into the culture media were precipitated by trichloroacetic acid and then digested with trypsin. The peptides were labeled with dimethyl labeling and analyzed by reversed phase nanoscale liquid chromatography coupled to a quadrupole Orbitrap mass spectrometer. From a total of 1508 identified proteins, 109 were differentially regulated, of which 108 were genuinely secreted. Factors significantly downregulated in hypoxic conditions included adiponectin, a known adipokine implicated in metabolic processes, as well as thrombospondin-1 and -2, and matrix metalloproteinase-11, all multifunctional proteins involved in extracellular matrix (ECM) homeostasis. Findings were validated by Western blot analysis. Expression studies of the relative genes were performed in parallel experiments in vitro, in differentiated 3T3-L1 adipocytes, and in vivo, in fat tissues from obese versus lean mice. Our observations are compatible with the concept that hypoxia may be an early trigger for both adipose cell dysfunction and ECM remodeling.

Dr. A. E. Laria, Dr. S. Messineo, Dr. B. Arcidiacono, Dr. E. Chiefari, Prof. D. Russo, Prof. A. Brunetti, Prof. D. P. Foti
Department of Health Sciences
University "Magna Græcia" of Catanzaro
Catanzaro, Italy
E-mail: brunetti@unicz.it; foti@unicz.it
Dr. M. Varano, Prof. G. Cuda, Prof. M. Gaspari
Department of Experimental and Clinical Medicine
University "Magna Græcia" of Catanzaro
Catanzaro, Italy
E-mail: gaspari@unicz.it
Prof. R. K. Semple, Dr. N. Rocha
Wellcome Trust-MRC Institute of Metabolic Science
University of Cambridge Metabolic Research Laboratories
Cambridge, UK
Prof. R. K. Semple, Dr. N. Rocha
The National Institute for Health Research Cambridge Biomedical Research Centre
Cambridge, UK
Prof. R. K. Semple
University of Edinburgh Centre for Cardiovascular Science
Edinburgh, UK

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1. Introduction

Obesity predisposes to the risk of type 2 diabetes mellitus, cardiovascular disease, and even some types of tumors. Its alarming prevalence has become a serious health problem in many countries in the world.[1,2] Studies in the last two decades have strongly implemented our knowledge about the biological role of adipose tissue, demonstrating that, besides storing lipids, this tissue actively participates in the regulation of important physiological processes, such as energy balance and glucose homeostasis, by secreting a variety of biomolecules collectively termed as adipokines, whose discovery and characterization are still ongoing.[3,4] Many studies have demonstrated that the adipose cell becomes dysfunctional in the obese state, and, as a consequence, the profile of the secreted adipokines changes towards an unfavorable pattern, that promotes insulin resistance, inflammation, endothelial dysfunction and thrombophilia.[5–7] Reversibility of obesity-related conditions is proved by changes in the biochemical profile following weight loss.[8]

Several studies support the concept that adipose tissue hypoxia (ATH) is an important trigger of adipose cell dysfunction in obesity. Although evidences are stronger in animal models than in humans, ATH in obesity is demonstrated by a series of mechanisms, including a reduction in adipose tissue blood flow, low availability of oxygen for clusters of adipose cells distant from the vasculature, reduced oxygen consumption, as well as mitochondrial dysfunction in adipose cells.[9–11] Thus, investigations aimed at identifying molecules induced or repressed by hypoxia in appropriate experimental models may be important to better understand the pathophysiological events linked to obesity. While the hypoxia-inducible factors (HIFs) have been recognized as the main molecular regulators of the cellular response to hypoxia,[12,13] many studies employing traditional molecular biology techniques have identified hypoxia target genes and gene products secreted by adipose cells, such as visfatin, vascular endothelial growth factor (VEGF), leptin, adiponectin, and other adipokines.[9,14–17] On the other hand, animal transgenic

Significance of the study

Obesity is a major health problem worldwide, in which adipose tissue expansion and dysfunction predispose to the risk of several illnesses, including type 2 diabetes mellitus and cardiovascular disease. As hypoxia has emerged as an early factor that triggers adipose tissue dysfunction in obesity, differentially expressed proteins were analyzed, by LC/MS/MS, in the secretome from hypoxic versus normoxic in vitro 3T3-L1 murine differentiated adipose cells as a surrogate biological system for adipose cell function/dysfunction studies. Taken together, in hypoxia, the identified protein changes were functionally coherent with extracellular matrix remodeling. Also, novel regulated proteins associated with the adipocyte secretome have been identified for the first time, and their roles deserve further investigation. Results were further validated by Western blot analysis and gene expression studies in 3T3-L1 cells, and notably, to some extent, also in adipose tissues from obese vs lean mice. In conclusion, the identified proteins in this study may be involved in early changes linked to adipose cell dysfunction, and may have the potential to be relevant to the pathophysiology of human obesity.

models constitutively overexpressing HIF-1α have contributed to the definition of hypoxia-mediated effects on proteins involved in insulin resistance and adipose tissue fibrosis.[18]

Proteomic technology has emerged as a powerful, and useful tool to identify key factors in the adipose proteome, and gain further insights into adipose cell biology.[19–21] In this context, by using different proteomic profiling approaches, the analysis of the adipose secretome has been adopted to analyze large sets of secreted proteins.[19–24] Investigations aimed at understanding the differential expression of the adipocyte secretome after cell stimulation with insulin, insulin sensitizers, or other agents have been already successfully undertaken,[25–27] as well as studies analyzing the secretome from human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes and adipocytes, exposed to the hypoxia-mimetic cobalt chloride ($CoCl_2$),[28] and exosomes from murine 3T3-L1 adipocytes under hypoxic conditions.[29]
Murine 3T3-L1 adipocytes are the most widely used cells for studying adipogenesis and obesity-related characteristics[30]; however, the effects of hypoxia on the whole secretome of 3T3-L1 adipocytes have not been investigated yet by a proteomic approach. To better understand the dynamic changes affecting adipose tissue secretion during hypoxia as a paradigm of the obese state, in the present work we comparatively analyzed the secretome of 3T3-L1 adipocytes, exposed to either normoxia or hypoxia. By using reversed-phase nanoscale liquid chromatography coupled to a quadrupole Orbitrap mass spectrometer, we identified 108 genuinely secreted, differentially expressed proteins, out of which only 28 where previously described by similar studies in the secretome from human and murine adipocytes.[28,29] Altogether, the role of the identified proteins appears to be important in the extracellular matrix (ECM) remodeling and relevant to adipose tissue dysfunction.

2. Experimental Section

2.1. Cell Culture, Viability, and Functional Tests in Normoxia and Hypoxia

3T3-L1 preadipocytes were grown and differentiated into adipocytes as previously described.[31] Once complete differentiation was reached, cells were maintained in medium without serum and antibiotics, in either normoxic (21% O_2) or hypoxic (2% O_2) conditions for 24 h. Cell viability in serum-free media was tested by 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[32] Lipid droplet accumulation in normoxic and hypoxic 3T3-L1 adipocytes was tested by red oil O staining, as reported by the manufacturer (Bio-Optica, Milan, Italy). Tests are detailed in Supporting Information A.

2.2. Immunoblotting

Western blot (WB) analyses were performed with standard techniques using nuclear extracts obtained from differentiated 3T3-L1 cells, and murine fat tissues, as previously described.[33] Proteins from 3T3-L1 secretome were loaded after trichloroacetic acid (TCA) precipitation. Antibodies used and densitometric analysis are reported in Supporting Information A.

2.3. Collection and Processing of Conditioned Media

Conditioned media from 3T3-L1 adipocytes, in either normoxic or hypoxic condition for 24 h, were collected and filtered through a 0.45 μM filter to remove cell debris. To concentrate samples, 10% v/v TCA was added and proteins precipitated overnight at 4 °C. Samples were centrifuged at 14 000 rpm for 30 min and the supernatants removed. After two washes with 200 μL cooled ether and 200 μL cooled acetone and centrifugation at 14 000 rpm for 10 min, 4 °C, supernatants were removed and dry samples resuspended in 50 μL Urea Buffer (6 M urea, 100 mM Tris, pH 8.0). The final protein concentration was measured using the Micro Bicinchoninic Acid (BCA) Protein Assay (G-Biosciences).

2.4. Trypsin Digestion, Dimethyl Labeling, and Peptide Fractionation

100 μg of each sample were reduced with 5 μL of 100 mM dithiothreitol (DTT) for 1 h at 37 °C, then alkylated with 6 μL of 24 mM iodoacetamide (IAA) by incubating 1 h at 37 °C in the dark to protect reduced cysteines. Finally, the excess of IAA was quenched by adding 1 μL of 2 mM DTT and the reaction allowed to proceed for 20 min at 37 °C. Solution was brought to a volume of 150 μL with HPLC-grade water and protein digestion performed overnight by adding 50:1 (w/w) trypsin (protein:enzyme ratio). After digestion, purification of 15 μg of peptides from each sample by C18 StageTips (EmporeTM-3M, C18 Extraction Disks) was performed as previously described.[34] Peptides were eluted in 20 μL of 50% acetonitrile and 0.1% formic acid and subsequently dried

by a speed vacuum system. For quantitative mass spectrometry analysis, dried samples were resuspended in 100 μL of 100 mM tetraethylammonium bicarbonate (TEAB). 4 μL of 4% CH_2O, and 4 μL of 4% CD_2O were added in order to label the samples with the "light" (L) form, and with the "medium" (M) form of the reagent, respectively. Four microliters of 0.6 M $NaBH_3CN$ were added to L and M labeled samples before incubating for 1 h at room temperature. The reaction was stopped by adding 16 μL of 1% w/v ammonia solution. The analysis was performed in biological duplicates. Each biological replicate was analyzed in forward/reverse labeling mode (L:M and M:L). Before performing LC-MS/MS analysis, samples were mixed in pairs and purified by C18 StageTips. Samples were purified and fractionated by Strong Cation Exchange Chromatography (SCX) using extraction disks (Empore Cation 47 mm Extraction Disks) as previously described.[34] Six fractions were obtained by eluting samples at six different concentrations of ammonium acetate (50, 75, 100, 150, 250, and 500 mM). All six eluents contained 20% acetonitrile v/v and 0.5% acetic acid v/v, except the last eluent, to which no acid was added.

2.5. Mass Spectrometry and Data Analysis

Peptides were analyzed by reversed phase nanoscale liquid chromatography coupled to a quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Odense, Denmark).

Peptides were analyzed by a nanoLC capillary column (75 μm i.d., length: 10 cm), packed with 3 μm C_{18} silica particles (Dr. Maisch GmbH, Ammerbuch, Germany). Peptides were loaded at 500 nL min^{-1} into the analytical column. Mobile phase A was 0.1% formic acid, 2% acetonitrile, whereas mobile phase B was 0.1% formic acid, 80% acetonitrile. The flow rate was 300 nL min^{-1}, and peptides were separated using a linear gradient of phase B from 8 to 35% in 55 min, and from 30 to 100% in 8 min. MS detection was performed on a Q-Exactive quadrupole ion trap-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). MS acquisition was performed in positive ionization mode, by nanoelectrospray (nESI). The acquisition of product ion mass spectra was done using the data-dependent acquisition (DDA) modes. Full scan m/z range was 350–1800. DDA was performed by using a top-12 method with resolution for full MS of 70 000 and of 17 500 for MS/MS; AGC target was 10^6 for full MS and 10^5 for MS/MS; maximum injection time (ms) was 50 ms for full MS, and 60 ms for MS/MS. Mass window for precursor ion isolation was 1.6 m/z. Normalized Collision energy was 25. Dynamic exclusion was 30 s. Raw data were processed for protein identification and quantitation using the software MaxQuant version 1.6.0.16,[35] using default parameters for dimethyl labeled peptides, with the following exceptions: minimum peak length: 3; minimum ratio count: 1 unique + razor peptide. Tandem mass spectrometry data were searched by the Andromeda search engine by querying the Mouse Reference Proteome Database (51 949 sequences, accessed on October 2017). Forward/reverse experiments for each biological replicate were searched as a single experiment in MaxQuant.

2.6. Animal Studies

Male C57BL/6JolaHsd mice were subjected to either normocaloric diet ($n = 5$) or high-fat diet ($n = 4$) for 18 weeks. Further details are described in Supporting Information A. After euthanasia by cervical dislocation, epididimal fat pads were excised and immediately stored in liquid nitrogen until use for RNA and protein extractions. The study was carried out in accordance with the Italian rules (D.M. 116/92) and the Directive 2010/63/EU of the European Parliament and of the European Council regarding the protection of animals used for scientific purposes, and with the permission of national authorities (MIN.SAL. N°114/2006-A 08/09/2006).

2.7. RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was extracted from adipocytes and from visceral fat tissues, as previously described.[36] Reverse transcription was performed from 1 μg RNA (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems, Thermo Fisher Scientific, Bremen, Germany). Primers used were designed according to sequences from the Gene Bank Database and are described in Supporting Information A. Quantitative real-time PCR (q-RT PCR) was performed by a real-time thermocycler (Eppendorf Mastercycler ep realplex ES), as previously described.[37] SYBR Green fluorescence was measured (Real Master Mix Sybr Rox 5 prime, Eppendorf, Hamburg, Germany) and relative quantification was made using *RPS9* to normalize the expression of the genes of interest. PCR reactions were repeated for three biological replicates.

2.8. Statistical and Bioinformatic Analysis

The data matrix produced by MaxQuant was uploaded in Perseus, version 1.6.07[35] for statistical analysis. After removing hits associated to contaminants and reverse database, data were log2-transformed. Protein hits quantified in at least three biological replicates were retained. After data normalization on protein median, a one-sample t-test was performed. Proteins whose average fold change was either > 2 or < 0.5, with associated p-value < 0.05, were considered differentially expressed.

Functional annotation of the significantly altered proteins was achieved using DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources 6.7.[38] Statistical significance was expressed as p-values after Benjamini–Hochberg correction.

Statistical analysis of expression studies was carried out using GraphPad software. The analysis of gene expression was performed using the Mann–Whitney test. Differences were considered significant when $p < 0.05$.

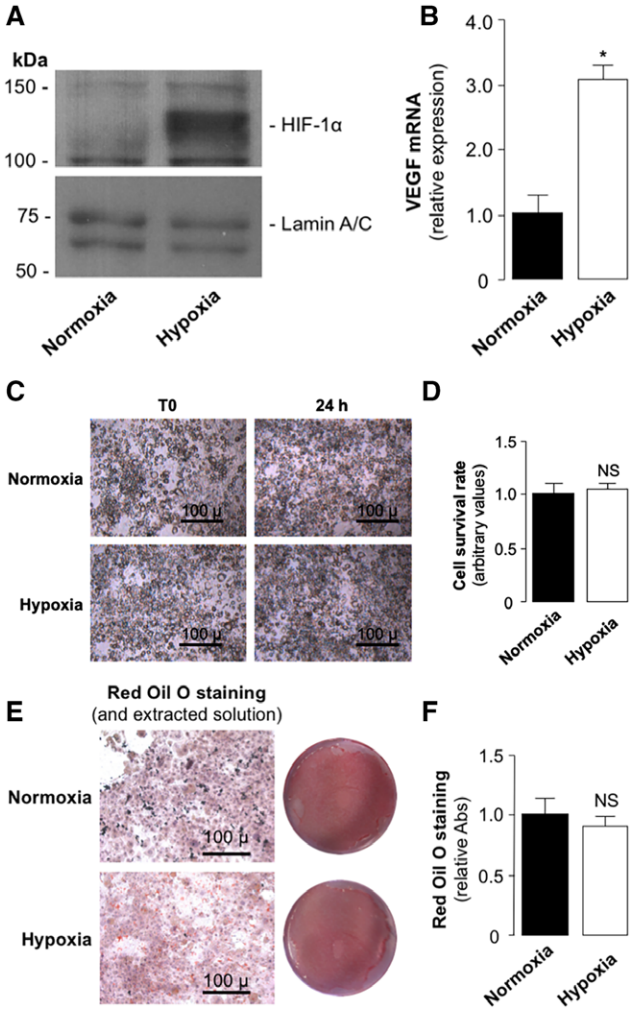

Figure 1. Induction of hypoxia in differentiated 3T3-L1 cells. A) Cells were incubated under normoxia or hypoxia (O_2, 2%) for 24 h, and Western blot (WB) for HIF-1α was carried out from nuclear protein extracts; lamin A/C, control of protein loading. B) q-RT PCR for *VEGF* gene expression was performed in parallel experiments. Data are means ± SEM of three independent experiments. *$p < 0.001$ versus normoxia. C) Microscopic images of 3T3-L1 adipocytes, before (T0) and after 24 h of normoxia and hypoxia, and relative MTT assay, D) after 24 h. E) Red Oil O staining of 3T3-L1 adipocytes and extracted solution after 24 h normoxia or hypoxia, and its absorbance (Abs), (F) at 490 nm. In (D) and (F), data are expressed as relative fold change compared to normoxia, which is assigned an arbitrary value of 1; data are means ± SEM of three independent assays. NS = not significant.

3. Results

3.1. Conditioned Media and MS Analysis Work Flow

To verify the appropriateness of the experimental conditions, differentiated 3T3-L1 adipocytes were incubated under normoxia or hypoxia for 24 h. Hypoxic conditions were confirmed by increased protein levels of HIF-1α, the hypoxia-inducible monomer of HIF-1, and by increased gene expression of *VEGF*, a known HIF-1 target gene (**Figure 1**A,B). No significant differences were detectable in cell viability in normoxia versus hypoxia, as measured by MTT assay (Figure C and D), and in mature adipocyte functions, such as lipid droplet accumulation, as visualized by red oil O staining (Figure 1E,F).

After 24 h, conditioned media from normoxic or hypoxic 3T3-L1 adipocytes, obtained in biological quadruplicates, were collected, and proteins precipitated by TCA, reduced with DTT, alkylated by IAA, and finally digested by trypsin. Trypsin-digested peptides were chemically labeled by "stable isotope dimethyl labeling," and samples analyzed by nLC-MS/MS. Whole secretome analysis led to the identification of 1508 proteins quantified in at least three of the four biological replicates (Supporting Information, Table 1).

Based on gene ontology, proteins classified as "extracellular region," "extracellular space," and "extracellular exosome" were considered genuinely secreted (Supporting Information B). Eight hundred and thirty-nine out of the 1508 identified proteins (56% of total proteins) were assigned to at least one of these three categories. The major molecular functions correlated with the identified proteins were related to protein, RNA, and nucleotide binding, while among the involved pathways, the metabolic pathways were predominant (Supporting Information B).

3.2. Quantitative Differential Expression and Classification of Proteins from 3T3-L1 Adipocyte Secretome in Hypoxia

In total, 109 identified proteins were differentially expressed (average fold change either > 2 or < 0.5) in the secretome from 3T3-L1 adipocytes following 24 h hypoxia. All proteins passing the criteria of significance and fold change were downregulated (Supporting Information, Table 2), indicating that hypoxia plays a regulatory role in the dynamic changes that affect the adipocyte secretome. Bioinformatic analysis revealed that more than 86% (94 out of 109) of the downregulated proteins were classified as belonging to either "extracellular region" (88 proteins) or "extracellular space" (69 proteins) (Supporting Information C). By including proteins assigned to the category "extracellular exosomes„ the total number of downregulated proteins classified as secreted was 108 (99%). Interestingly, the background of non-secreted proteins, likely deriving from cell leakage, was much higher when considering the entire set of the identified proteins. In fact, proteins classified as either belonging to "extracellular region," "extracellular space," or "extracellular exosome" were 839 in total (56% of the 1508 identified proteins) (Supporting Information B). The most prominent biological processes affected by hypoxia-induced protein downregulation were cell adhesion, ECM organization, proteolysis, collagen fibril organization, all of which paradigmatic of a precocious remodeling of ECM in response to hypoxia. Among the functions related to the identified proteins, calcium and heparin binding were the most prominent, while the main affected pathways were related to ECM receptor interaction, focal adhesion, and to the involvement of the phospatidylinositol-3 kinase-AKT signaling (Supporting Information C).

Notably, when compared with previous reports investigating hypoxia-regulated proteins in the secretome from human SGBS adipocytes[28] or in exosomes from 3T3-L1 adipocytes,[29] our

Table 1. Differentially expressed proteins in the secretome from 3T3-L1 adipocytes in hypoxic/normoxic conditions.

Uniprot accession	Gene	Average fold change[a)]	Extracellular region	Extracellular space	Extracellular exosomes	↓ Proteins in 3T3-L1[29]	↓ Proteins in SGBS[28]
Q61646	**Hp**	0.06	Yes	Yes	Yes		
Q91XL1	**Lrg1**	0.09		Yes	Yes		
P03953	**Cfd**	0.13	Yes	Yes	Yes		
P34928	**Apoc1**	0.14	Yes		Yes		
P01027	C3	0.17	Yes	Yes	Yes	Yes	
P11152	Lpl	0.18	Yes	Yes	Yes	Yes	
P47879	**Igfbp4**	0.18	Yes	Yes			
Q9R1Q9	**Atp6ap1**	0.19			Yes		
E9QPB2	**Sbsn**	0.20	Yes		Yes		
P11859	**Agt**	0.21		Yes	Yes		
P70663	**Sparcl1**	0.21	Yes	Yes	Yes		
Q9D7I0	**Shisa5**	0.21			Yes		
Q9JK53	**Prelp**	0.21	Yes	Yes	Yes		
Q99P87	**Retn**	0.22	Yes	Yes	Yes		
Q07797	Lgals3bp	0.23	Yes	Yes	Yes	Yes	
A0A0N4SVV4	**Rarres2**	0.24	Yes		Yes		
P08121	Col3a1	0.24	Yes	Yes			Yes
P08226	Apoe	0.24	Yes	Yes	Yes		Yes
Q01149	Col1a2	0.24	Yes	Yes	Yes	Yes	Yes
Q06890	**Clu**	0.24	Yes	Yes	Yes		
Q61581	**Igfbp7**	0.24	Yes	Yes	Yes		
Q8R2G6	**Ccdc80**	0.24	Yes				
P11087	Col1a1	0.25	Yes	Yes		Yes	Yes
P21460	Cst3	0.25	Yes	Yes	Yes		Yes
Q61398	Pcolce	0.25	Yes	Yes	Yes		Yes
Q9DD06	**Rarres2**	0.25	Yes		Yes		
P28653	**Bgn**	0.26	Yes		Yes		
Q03350	**Thbs2**	0.26	Yes				
Q60994	**Adipoq**	0.26	Yes	Yes	Yes		
G3 × 9T8	**Cp**	0.27	Yes	Yes	Yes		
P07214	Sparc	0.27	Yes	Yes			Yes
Q04857	Col6a1	0.27	Yes		Yes	Yes	Yes
Q62000	**Ogn**	0.27	Yes	Yes	Yes		
Q8BND5	**Qsox1**	0.27	Yes	Yes	Yes		
Q9R1E6	**Enpp2**	0.27	Yes	Yes			
O09164	**Sod3**	0.28	Yes	Yes	Yes		
P55065	**Pltp**	0.28	Yes	Yes			
Q02788	Col6a2	0.28	Yes	Yes	Yes	Yes	Yes
Q9EPL2	**Clstn1**	0.28	Yes		Yes		
O88207	Col5a1	0.29	Yes		Yes		Yes
P07759	**Serpina3n**	0.29	Yes	Yes	Yes		
P51910	**Apod**	0.29	Yes	Yes	Yes		
Q9QZZ6	**Dpt**	0.29	Yes	Yes	Yes		
A2AVA0	**Svep1**	0.30	Yes				
P06909	**Cfh**	0.30	Yes	Yes	Yes		
P43025	**Clec3b**	0.30	Yes	Yes	Yes		
P10605	Ctsb	0.31	Yes	Yes	Yes		Yes
Q07235	**Serpine2**	0.31	Yes	Yes			

(*Continued*)

Table 1. Continued.

Uniprot accession	Gene	Average fold change[a)]	Extracellular region	Extracellular space	Extracellular exosomes	↓ Proteins in 3T3-L1[29]	↓ Proteins in SGBS[28]
Q3UQ28	**Pxdn**	0.31	Yes	Yes	Yes		
E9PWQ3	Col6a3	0.32		Yes	Yes	Yes	
P97298	Serpinf1	0.32	Yes	Yes	Yes		Yes
Q99K41	Emilin1	0.32	Yes		Yes	Yes	
Q9JM58	**Crlf1**	0.32	Yes				
P01029	**C4b**	0.33	Yes	Yes	Yes		
P01887	**B2m**	0.33	Yes	Yes	Yes		
P02463	Col4a1	0.33	Yes				Yes
P08122	**Col4a2**	0.33	Yes		Yes		
P97873	**Loxl1**	0.33	Yes	Yes			
Q61554	Fbn1	0.33	Yes	Yes	Yes		Yes
Q9WVJ9	**Efemp2**	0.33	Yes		Yes		
P28654	**Dcn**	0.34	Yes	Yes			
Q02853	**Mmp11**	0.34	Yes				
Q3U962	Col5a2	0.34	Yes				Yes
Q61112	**Sdf4**	0.34			Yes		
Q62356	**Fstl1**	0.34	Yes	Yes	Yes		
Q8QZR4	**Oaf**	0.34			Yes		
Q62009	**Postn**	0.35	Yes	Yes			
J3QQ16	Col6a3	0.36		Yes	Yes	Yes	
O08538	**Angpt1**	0.36	Yes	Yes	Yes		
P51885	Lum	0.36	Yes	Yes	Yes	Yes	
Q08879	**Fbln1**	0.36	Yes	Yes	Yes		
Q8CG16	**C1ra**	0.36	Yes		Yes		
P30412	**Ppic**	0.37			Yes		
Q8BS97	**Vcan**	0.37	Yes	Yes			
Q8CG14	**C1s1;C1sa;C1sb**	0.37	Yes		Yes		
Q9WVH9	**Fbln5**	0.37	Yes	Yes	Yes		
P10493	Nid1	0.38	Yes		Yes	Yes	
P25785	**Timp2**	0.38	Yes	Yes	Yes		
Q61207	**Psap**	0.38	Yes	Yes	Yes		
Q9R0E1	**Plod3**	0.39			Yes		
P33434	Mmp2	0.40	Yes	Yes			Yes
O88393	**Tgfbr3**	0.41	Yes	Yes	Yes		
P35564	**Canx**	0.41			Yes		
P11276	Fn1	0.42	Yes	Yes	Yes	Yes	Yes
Q00493	**Cpe**	0.42	Yes	Yes	Yes		
Q06335	**Aplp2**	0.42			Yes		
Q9WVB4	**Slit3**	0.42	Yes	Yes			
P12023	**App**	0.43		Yes	Yes		
P28301	**Lox**	0.43	Yes	Yes			
Q61508	**Ecm1**	0.43	Yes	Yes	Yes		
Q62165	**Dag1**	0.43	Yes	Yes	Yes		
P35441	Thbs1	0.44	Yes	Yes	Yes	Yes	
P97857	**Adamts1**	0.44	Yes				
Q02819	**Nucb1**	0.44	Yes	Yes	Yes		
Q60648	**Gm2a**	0.44			Yes		
Q9R0E2	**Plod1**	0.44			Yes		
O08746	**Matn2**	0.45	Yes				

(*Continued*)

Table 1. Continued.

Uniprot accession	Gene	Average fold change[a)]	Extracellular region	Extracellular space	Extracellular exosomes	↓ Proteins in 3T3-L1[29]	↓ Proteins in SGBS[28]
Q9CQF9	**Pcyox1**	0.45			Yes		
E9PZ16	Hspg2	0.46	Yes	Yes	Yes	Yes	
P16675	**Ctsa**	0.46			Yes		
P37889	**Fbln2**	0.46	Yes		Yes		
P48759	**Ptx3**	0.46	Yes	Yes			
Q9JJH1	**Rnase4**	0.46	Yes		Yes		
Q9WVJ3	**Cpq**	0.46	Yes	Yes	Yes		
P70699	**Gaa**	0.48			Yes		
Q99JI6	**Rap1b;Rap1a**	0.48			Yes		
O08992	Sdcbp	0.49	Yes	Yes	Yes	Yes	
P06797	**Ctsl**	0.49		Yes	Yes		

a) Fold change <1 indicates downregulation in hypoxic conditions.
Bold refers to newly identified regulated proteins, as compared with two previous reports in 3T3-L1[29] and SGBS[28] adipocytes.

Table 2. Differentially expressed proteins in the secretome from 3T3-L1 adipocytes as verified by Western blot (WB) analysis.

Accession	Protein description	Gene name	Peptides	Average fold change[a)]	*p*	WB
Q60994	Adiponectin	Adipoq	11	0.26	0.00	Confirmed
P35441; Q80YQ1	Thrombospondin-1	Thbs1	33	0.44	0.01	Confirmed
Q03350	Thrombospondin-2	Thbs2	29	0.26	0.00	Confirmed
Q02853	Stromelysin-3	Mmp11	2	0.34	0.01	Confirmed

a) Fold change <1 indicates downregulation in hypoxic conditions.

findings showed that of the 108 secreted, differentially expressed identified proteins, 28 had been previously detected in at least one of the above-mentioned studies, whereas 80 (74%) were newly identified, regulated proteins (**Table 1**).

3.3. Western Blots and Gene Expression Studies Relative to Candidate Proteins in 3T3-L1 Cells

Among the 109 differentially expressed proteins emerged from LC-MS/MS experiments, candidates were selected for further validation by WB analyses (**Table 2**) and subsequent gene expression studies. Selection criteria for candidate proteins have considered the potential functional significance of the identified proteins in the context of obesity-related disorders, our availability of specific antibodies, and the lack of data about differential expression induced by hypoxia for many proteins.

Therefore, adiponectin, thrombospondin-1 (THBS1) and -2 (THBS2), as well as matrix metalloproteinase 11 (MMP-11, also known as stromelysin-3) were chosen among the downregulated proteins. WB analysis confirmed results obtained in LC-MS/MS experiments (Table 2 and **Figure 2**), as all the four proteins tested were shown to be downregulated in hypoxia. In hypoxic versus normoxic 3T3-L1 cells, the decrease in adiponectin, THBS1 and THBS2 protein species was confirmed by q-RT PCR analysis of the respective mRNAs, while MMP-11 gene expression was upregulated in hypoxia (Figure 2).

3.4. Gene Expression Studies Relative to Candidate Proteins in Obese Versus Lean Mice

To investigate whether gene expression findings obtained in vitro, in differentiated 3T3-L1 cells, were replicated in vivo, we compared results from gene expression studies in fat tissue from obese versus lean mice. As expected, in fat specimen from obese mice, a higher detectable expression of HIF-1α was observed, thereby confirming ATH, that was compatible with the excess of body weight in these animals (Supporting Information A, Figure S1). Differences in gene expression between hypoxic versus normoxic fat tissues (**Figure 3**) paralleled findings obtained in vitro, in 3T3-L1 adipocytes, only to a certain extent. This discrepancy may account for the complex functional interactions of the different cells within fat tissue, and/or for systemic effects.

4. Discussion

Hypoxia is considered an important trigger of changes that occur in the adipose tissue during obesity. In this work, we comprehensively profiled, under normoxic and hypoxic conditions, the secretome of murine differentiated 3T3-L1 adipose cells, a well-characterized cell line widely used in studies of adipose cell biology. Proteomic analysis identified 109 differentially expressed proteins, all of which were downregulated by hypoxia. This peculiar finding can be explained by the inhibitory effects of

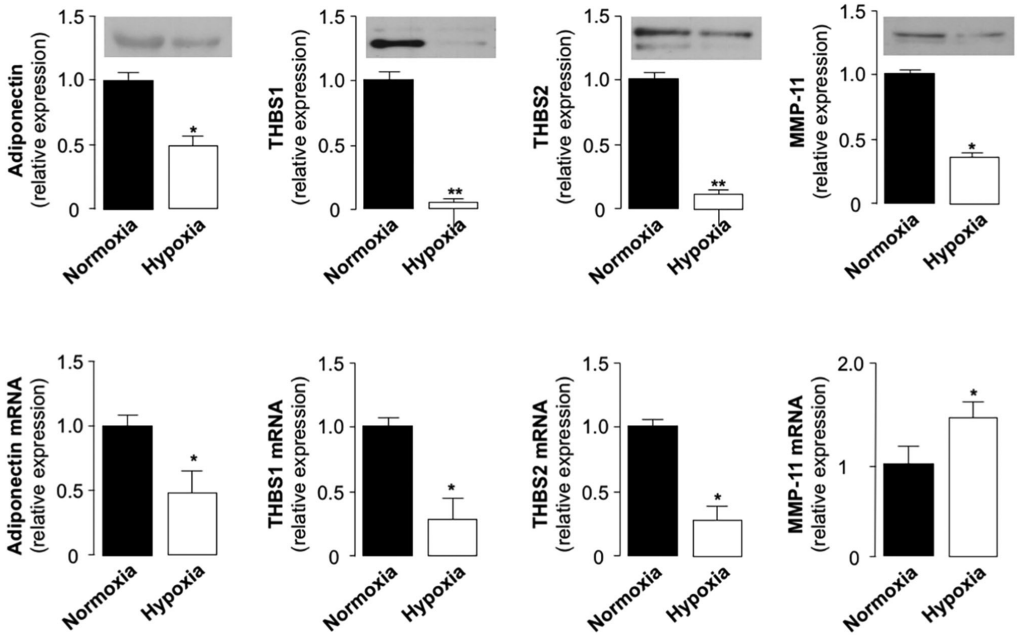

Figure 2. Protein and gene expression results of the hypoxia-regulated selected factors in 3T3-L1 adipocytes. Cells were incubated under normoxia or hypoxia for 24 h. TCA- precipitated secretome proteins were analyzed by WBs (upper), whereas q-RT PCRs (lower) were performed contextually, from cells under the same experimental conditions. Data are means ± SEM from at least three separate experiments. Densitometric analysis of WBs is shown in bar graphs. Data are expressed as arbitrary units, with normoxia = 1 in each assay. Representative WBs are shown. *$p < 0.05$ versus normoxia, **$p < 0.001$ versus normoxia.

hypoxia on protein synthesis, as already stated elsewhere.[28] When compared with previous similar reports,[28,29] we identified 80 novel secreted proteins, that were downregulated in hypoxic conditions. Possible explanations for the discrepancy between our results and those from similar works could be: i) the broader proteome coverage obtained by the newer-generation instrumentation used in this study; ii) the stringent criteria adopted in our analysis for the definition of hypoxia-regulated proteins; iii) the high prevalence, among the regulated proteins, of truly secreted proteins; iv) differences between cell models (human vs. murine adipose cells). Most of these considerations, indeed, represent an added value of this work. Bioinformatic analysis of the hypoxia-induced differentially expressed proteins have shown that following hypoxia, 3T3-L1 adipocytes precociously respond by reorganizing and remodeling ECM. These results integrate and are in line with previous findings obtained in transgenic animal models expressing HIF-1α,[18] and in proteomic studies using the HIF-1α chemical inducer, $CoCl_2$, in human SGBS pre- and adipose cells,[28] both of which underlie the role of ECM reorganization in the early events linked to ATH.

Among the downregulated proteins, we identified and further analyzed adiponectin, THBS1, THBS2, and MMP-11. Adiponectin, THBS1, THBS2, but not MMP-11 had been previously identified in human adipocyte secretome by proteomic strategies.[28] However, with the exception of THBS1,[29] none of these proteins had been described as differentially regulated by hypoxia by using similar approaches. Adiponectin is well known to regulate glucose levels and fat metabolism, and to play a role in insulin sensitivity. Its reduction has been described in obesity, type 2 diabetes, and atherosclerosis.[39] THBS1 and THBS2 are trimeric, multifunctional proteins, that

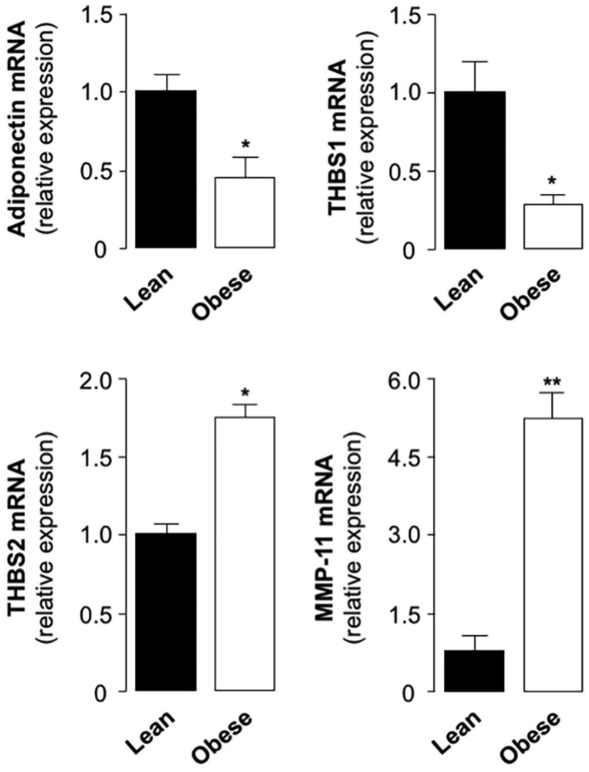

Figure 3. Gene expression studies (q-RT-PCR) of the hypoxia-regulated selected factors in epididymal fat from obese versus lean mice ($n = 4$ and 5, respectively). Data are from three separate assays and expressed as arbitrary units, with data from controls (lean) = 1 in each group. *$p < 0.05$, **$p < 0.001$.

influence cell-matrix interactions, and are differently expressed in various tissues. Unlike other proteins with a prominent structural role in ECM, these matricellular molecules bind specific membrane receptors, including CD36, CD47, and integrins, as well as cytokines, growth factors, and proteases.[40] Their functions include anti-angiogenic and wound healing effects, cell proliferation and differentiation, immune regulation, and activation of TGF-β. THBS1 has been reported to be expressed in adipose tissue, and to play a role in adipogenesis, although the knock-out of the relative gene in mice does not alter adipose tissue abundance, distribution, and vascularization.[41] In vitro studies have documented that THBS1 is downregulated by hypoxia in mouse embryonic fibroblasts.[42] However, the complexity of the interactions and tissue-specific expression of its receptors may account for the paradox of opposed effects described in the literature. THBS2 knockdown has been associated with abnormal collagen fibrillogenesis, and increased levels of pericellular metalloproteinase 2. MMP-11 belongs to the family of matrix metalloproteinases and its role in ECM remodeling is not well known, while its involvement in cancer progression has been demonstrated. We recently explored the expression of this protein in 3T3-L1 cells and in fat tissue from mice under low or high-fat diet, showing that this protein is overexpressed in insulin resistance.[43]

Altogether, the decreased expression of the above identified proteins by hypoxic adipose cells coherently leads to ECM remodeling. In this respect, it is intriguing that adipose tissue, being the main energy-storing compartment, has the unique peculiarity to naturally expand, without cell transformation, until oxygen diffusion limits, and this biological behavior is remarkably similar to solid tumor growth.[44] Discrepancies between in vitro and in vivo findings reported in this study may have several explanations, including the complexity of fat tissue cellular composition and molecular networks, and the persistent hypoxic conditions, that may implicate different adaptive responses.

Although the biology of murine and human obesity presents important differences, and whether rodent models fit for research of human obesity and related disorders remain a questionable issue,[45] 3T3-L1 adipose cells still represent a valuable tool to investigate obesity-related disorders. On the other hand, experiments in cell lines represent an oversimplification of what occurs in vivo, in whole animals, although they are often needed to establish "proof of principle." In this context, future studies may be undertaken to explore the role of the newly identified, hypoxia-regulated proteins and their link to adipose cell dysfunction in obesity.

We believe that, by providing information about the dynamic changes that occur in the adipocyte secretome in hypoxia, we may improve our knowledge about the pathophysiological events characterizing the transition to the obese state, as well as identify new biomarkers and/or therapeutic targets that could be searched for in obesity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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A.E.L. and S.M. contributed equally to this work. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[46] with the dataset identifier PXD006820.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

adipocytes, hypoxia/ LC-MS/MS, obesity, secretome

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